BIOCHEMICAL ASPECTS OF THE GROWTH OF FELINE PNEUMONITIS VIRUS IN THE CHICK EMBRYO YOLK SAC[1]

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The many unusual properties of the psittacosis-lymphogranuloma group of infectious agents have made their exact taxonomic position a matter of doubt (Rake, 1953; Meyer, 1953). However, whether these intracellular parasites are really viruses or rickettsiae or neither, there is no doubt that they are a large and important group of organisms lying close to the nebulous line which separates virus from not-virus.

For several years, my students and I have been studying some of the biochemical aspects of the growth of feline pneumonitis virus in chick embryo yolk sac. Feline pneumonitis virus is a typical member of the psittacosis-lymphogranuloma group and was first described by Baker (1942). Like other members of its group, this agent grows to a high titer in the yolk sac, and the very thin and easily separated yolk sac may be conveniently used both for investigating the relation of virus multiplication to host cell metabolism and for preparing purified virus suspensions.

In beginning, I should like to recall to you the cycle of development in host cells exhibited by feline pneumonitis virus and the other agents of the psittacosis group (Weiss, 1949). The basic infectious unit is the elementary body (figure 1). In electron micrographs of air-dried metal-shadowed preparations, the elementary body is about 0.4 micron in diameter (Hamre, Rake and Rake, 1947; Moulder and Weiss, 1951*a*) and exists in two different forms: one shows distinct central and peripheral components, whereas the other appears homogeneous. Both are probably infectious forms of the virus (Heinmets and Golub, 1948; Crocker, 1954). Virus growth is started when an elementary body enlarges into an initial body. The initial body then develops into a cluster of initial bodies, apparently by a series of binary fissions (Rake and Jones, 1942). The clusters finally form large vesicles packed with elementary bodies. About 48 hours after the host cell is infected, the vesicle bursts, ruptures the cell and releases a swarm of new elementary bodies to infect fresh cells (Weiss, 1949).

When feline pneumonitis virus is inoculated into the yolk sac of a 6 day chick embryo, the demonstrable virus titer falls sharply during the first few hours after infection and does not rise for about 24 hours. The work of other investigators (Sigel *et al.*, 1951) suggests the presence of noninfectious virus in this interval. After 24 hours, new infectious units rapidly appear, and the virus titer rises at an increasing rate until the embryo dies between the fourth and fifth day of infection. Then the embryo dies, virus multiplication ceases, and the titer in the yolk sac slowly declines (Moulder and Weiss, 1951*b*; Moulder *et al.*, 1954). Just as the embryo dies, the virus reaches its maximum concentration, and almost all of the entodermal cells of the yolk sac are infected. One yolk sac contains approximately 10^8 LD_{50}'s of virus, and each LD_{50} is equivalent to about 25 elementary bodies (Moulder *et al.*, 1954). From data obtained in purification experiments, we have estimated that this amount of virus represents about one per cent of the fat-free dry weight of the yolk sac (Zahler and Moulder, 1953).

One of the most obvious ways to investigate the biochemical events associated with the reproduction of an intracellular parasite is to compare the biochemical properties of normal and infected tissue at different times during growth of the parasite. From the time chick embryos are infected with feline pneumonitis virus until they succumb to the infection, there is no difference in either the oxygen consumption (Moulder and Weiss, 1951*b*) or the concentration of organic phosphorus compounds (Zahler, 1953) in normal and infected yolk sac. Since feline pneumonitis virus never makes up more than one per cent of the active cell mass, it is not surprising that its multiplication causes no gross disruption in the metabolic pattern of the yolk sac.

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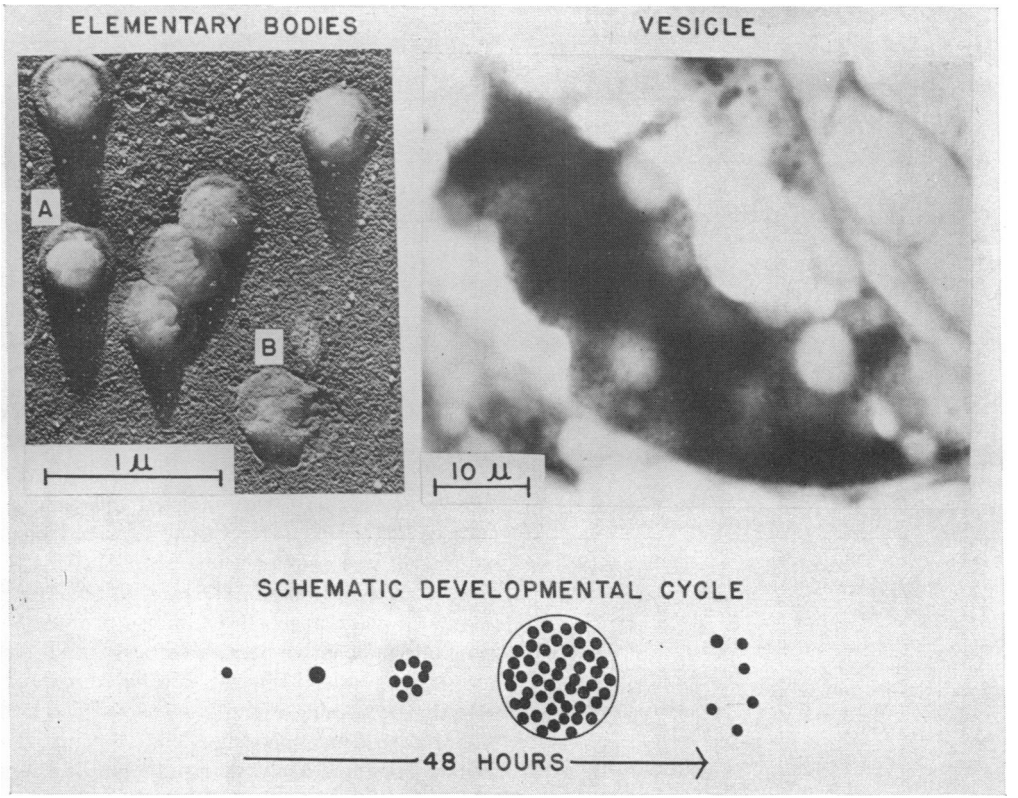

Figure 1. Developmental cycle of feline pneumonitis virus in the chick embryo yolk sac.

However, when the embryo is killed by infection and multiplication of the virus ceases, the oxygen consumption of infected yolk sac falls sharply (Moulder and Weiss, 1951*b*), and its organic phosphorus compounds are rapidly broken down (Zahler, 1953). This close chronological correlation between the cessation of virus growth and decrease in oxygen uptake and in organic phosphates suggested that a high level of yolk sac metabolism is necessary for virus growth and led to further study of the energy requirements for multiplication.

The yolk sac has a large store of endogenous substrates which it oxidizes at an almost undiminished rate for many hours. The low respiratory quotient, together with the large fat stores of the yolk sac, makes it likely that these substrates are long-chain fatty acids rather than carbohydrates. Since this oxidation is coupled with the uptake of inorganic phosphate into organic linkage, the yolk sac probably obtains its metabolic energy from high-energy phosphate compounds generated by the oxidation of its endogenous substrates (Moulder *et al.*, 1953).

We have been able to study the relation of energy metabolism to the multiplication of feline pneumonitis virus in a simple *in vitro* system (Moulder *et al.*, 1953). When the yolk sac is removed from a chick embryo on the third day of infection and incubated for 24 hours in Ringer-bicarbonate, the virus continues to multiply at a rate approaching that expected if the yolk sac had remained in the intact embryo. There is usually a 100–200-fold increase in virus titer in this period, representing about 99% of the total virus synthesized. The effect of different agents and conditions on virus multiplication *in vitro* is best explained by assuming that high energy phosphate bonds generated by the aerobic oxidation of the endogenous substrates supply the metabolic energy for virus growth (table 1). Thus, maximum virus multiplication occurs without addition of any oxidizable substrates; the virus grows only under aerobic conditions; and only those common metabolic poisons which strongly inhibit the energy metabolism of yolk sac, such as fluoride, azide and dinitrophenol, are also capable of inhibiting the growth of the

TABLE 1

Growth of feline pneumonitis virus in isolated yolk sac

Substance Added*	Concentration	Effect on Energy Metabolism	Effect on Virus Growth
	M		
Anaerobiosis	—	No inorganic P uptake	Complete inhibition
Glucose	0.01	None	None
Pyruvate	0.01	None	None
Fluoride	0.01	Inhibits O_2 uptake	Complete inhibition
Azide	0.001	Inhibits O_2 uptake	Complete inhibition
Malonate	0.01	None	None
Fluoroacetate	0.01	None	None
2,4-Dinitrophenol	0.0001	Inhibits inorganic P uptake	Partial inhibition

* Yolk sac incubated at 37 C and pH 7.4 with Ringer-bicarbonate and 5% CO_2-95% air.

virus. Malonate and fluoroacetate only slightly inhibit the oxygen uptake of yolk sac and do not interfere with virus multiplication. The behavior of the malonate may be contrasted with its effect on the growth of influenza virus in isolated chorioallantoic membrane: in this host-virus system, malonate inhibits the oxygen uptake of the host tissue and prevents virus multiplication (Ackermann, 1951).

Since phosphorus containing substances are involved in every phase of cell metabolism, we have searched for changes in the concentration and rate of synthesis and breakdown of organic phosphorus compounds within the infected cell, which might be attributed to the multiplication of feline pneumonitis virus (Zahler, 1953). As already mentioned, infection does not change the quantitative distribution of organic phosphorus within the yolk sac (table 2). These data are expressed in relation to the concentration of desoxypentose nucleic acid phosphorus since diploid cells of the same species all contain the same amount of desoxypentose nucleic acid.

The rate at which organic phosphates are broken down and synthesized may be estimated by measuring the incorporation of radioactive inorganic phosphate into the various fractions.

TABLE 2

Effect of feline pneumonitis virus on the phosphorus fractions of yolk sac

Fraction	Grams P in Fraction / Grams DNA P		Relative Specific Activity*	
	Normal	Infected	Normal	Infected
Acid-sol. P	13	13	100	100
Alc.-ether sol. P	13	12	6	9
Protein P	4	4	4	4
PNA P	5	5	21	46
DNA P	1	1	23	80

* In the presence of $H_3P^{32}O_4$ injected 24 hours before.

The radiophosphate was injected into infected embryos 24 hours before their expected death so that it was present while almost all of the virus was synthesized. The acid-soluble phosphorus rapidly reached equilibrium with the inorganic radioactive phosphate, and the specific activities of other fractions are expressed relative to the specific activity of the acid-soluble phosphorus. Infection with feline pneumonitis virus greatly increased the rate of synthesis and breakdown of the nucleic acid fractions. In particular, the desoxypentose nucleic acid almost reached apparent equilibrium with the acid-soluble phosphorus. The increase is not produced by nonspecific damage to the yolk sac and is the one change in host cell metabolism which can definitely be connected with multiplication of feline pneumonitis virus. Since the virus nucleic acid amounts to only about one per cent of the host nucleic acid, it is surprising that the synthesis of this small amount of virus nucleic acid causes such a great increase in the incorporation of radioactive phosphate into the nucleic acids of infected yolk sac.

Studies with purified virus have provided only a partial answer to this puzzle. Feline pneumonitis virus may be separated from infected yolk sac by digesting the normal tissue components with trypsin and adsorbing them on "celite". The purified virus suspensions show little non-virus material in the light and electron microscopes, migrate with a single boundary in the electrophoresis apparatus, and form no precipitate with antiserum against whole normal yolk sac (Moulder and Weiss, 1951*a*; Zahler and Moulder, 1953). Purified preparations contain

TABLE 3

Comparison of the uptake of $H_3P^{32}O_4$ into feline pneumonitis virus and into the yolk sac from which it was isolated

Fraction	Grams P in Fraction / Grams DNA P		Relative Specific Activity*	
	Yolk sac	Purified virus	Yolk sac	Purified virus
Acid-sol. organic P	12	8	100	14
Alc.-ether sol. P	25	14	9	100
Protein P	10	3	4	124
PNA P	3.5	2.5	46	410
DNA P	1.0	1.0	80	505

* Acid soluble organic P of yolk sac = 100. $H_3P^{32}O_4$ injected 24 hours before.

all five of the usual organic phosphate fractions (table 3). Two different kinds of experiments employing radioactive phosphate strongly suggest that all except the acid-soluble phosphate fraction are true virus components.

First, uninfected yolk sac highly labeled *in vivo* in all its phosphate fractions with radiophosphate was homogenized with infected, but unlabeled yolk sac. The virus was separated from the mixture, and the different phosphate fractions were tested for radioactivity. The total radioactivity of the purified virus was very small (not shown in table 3), indicating good separation of the virus from the phosphorus compounds of normal yolk sac (Zahler and Moulder, 1953).

Second, radiophosphate was injected into infected embryos, the yolk sacs were harvested at peak virus titer 24 hours later, and both the purified virus and the whole infected yolk sac were analyzed. With the exception of the acid-soluble phosphorus, which was probably nonspecifically adsorbed, all the virus phosphate fractions had many times the specific activity of the corresponding fractions of yolk sac (Zahler and Moulder, 1953). The high specific activity of the phosphorus of alcohol-ether soluble, the protein, and the nucleic acid fractions can hardly be explained except by assuming that they represent true virus constituents, synthesized after the radiophosphate was injected. It, therefore, appears that feline pneumonitis virus contains both pentose and desoxypentose nucleic acids—like bacteria and rickettsiae and unlike the smaller animal viruses which almost always contain only one type of nucleic acid. Crocker (personal communication) has also recently found that the closely related meningopneumonitis virus contains both nucleic acids. However, the total nucleic acid content is low, and the elementary bodies probably consist mainly of protein and fat.

The specific activities of the virus phosphate fractions fell into two groups: (a) the alcohol-ether soluble and protein phosphorus had specific activities equal to that of the whole infected yolk sac acid-soluble phosphorus at time of harvesting; (b) the two nucleic acid fractions had 4 to 5 times the specific activity of the yolk sac acid-soluble phosphorus. The only apparent explanation for the very high specific activity of the virus nucleic acid phosphorus is that it was incorporated into the virus soon after the radiophosphate was injected, while the specific activity of the yolk sac inorganic phosphate was very high. The phosphorus of the alcohol-ether soluble and protein fractions may have been taken up into the virus at a later time when the specific activity of the yolk sac inorganic phosphate had been diluted. Alternatively, all four fractions may have been synthesized at the same time, but the alcohol-ether soluble and protein phosphorus remained in equilibrium with the acid-soluble phosphorus of yolk sac, while the nucleic phosphorus did not. Further work is obviously needed for precise interpretation of these results.

In spite of their high specific activities, the virus nucleic acids account for only a portion of the increased uptake of inorganic phosphate into the total nucleic acids of infected yolk sac. It is conceivable that multiplication of the virus induces the synthesis of nucleic acids, and other substances as well, which do not appear in the final infective virus. In this connection, it is of interest that feline pneumonitis virus forms a hemagglutinin which may be completely separated from the infective elementary bodies (Gogolak, 1954).

One of the properties of the agents of the psittacosis-lymphogranuloma group which sets them apart from the other infectious agents usually called viruses is their susceptibility to a large number of the chemotherapeutic agents active against bacteria: sulfonamides, penicillin, chlortetracycline, oxytetracycline, and chloramphenicol (Eaton, 1950; Hurst, 1953). Although

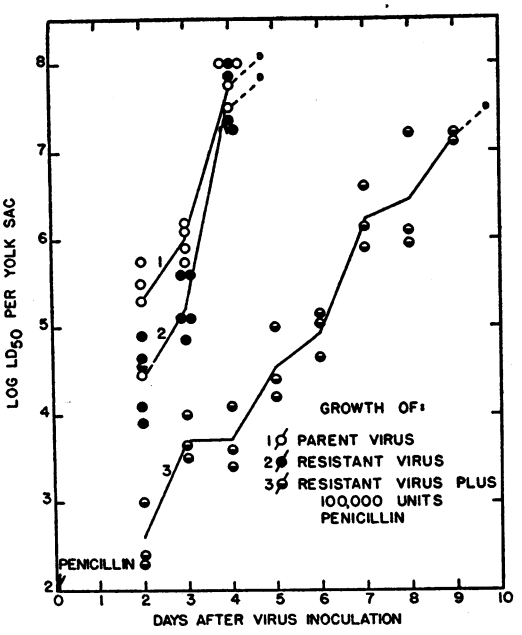

Figure 2. Growth rates of parent and penicillin resistant feline pneumonitis virus in the presence and absence of penicillin.

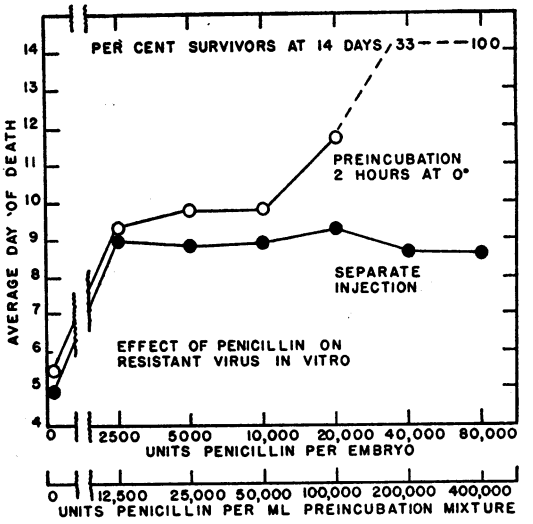

Figure 3. Effect of penicillin on penicillin resistant feline pneumonitis virus *in vitro*.

alternate explanations are conceivable, it seems most reasonable to assume that chemotherapeutic agents prevent the growth of the viruses of the psittacosis group just as they prevent the growth of bacteria, by inhibiting enzyme systems in the agents themselves.

Since penicillin interferes with both protein and nucleic acid metabolism in bacteria (Work, 1952), we have produced a penicillin resistant strain of feline pneumonitis virus in the hope that study of the properties of the resistant virus might lead to a better understanding of the mechanism of action of the antibiotic and eventually to a knowledge of the enzymatic capabilities of the virus (Moulder *et al.*, 1954). Benzyl penicillin, penicillin G, increases the survival rate of embryos infected with the parent strain of virus in proportion to the log of the amount of penicillin injected (Hamre and Rake, 1947; Eaton *et al.*, 1948; Weiss, 1950). A hundred units of penicillin protects over half of the infected embryos for at least 10 days; 10,000 units, almost all of them. With the resistant virus obtained after 33 chick embryo passages in the presence of increasing concentrations of penicillin, there is no such relationship between protection and penicillin dosage. Any amount of penicillin from 100 to 100,000 units per embryo produces the same 3 to 4 day delay in death, and all of the infected embryos die. This degree of resistance was maintained unchanged through 20 drugless chick embryo transfers. In the absence of penicillin, the parent and resistant viruses grow at the same rate (figure 2). When 100,000 units of penicillin are injected into each embryo immediately after virus inoculation, the parent virus never reaches a measurable titer, while the resistant virus grows at a rate one-half that achieved in the absence of penicillin and eventually reaches the same maximum virus titer. We have tentatively concluded that the penicillin resistant virus multiplies by the normal reproductive mechanism in the absence of the antibiotic, and that, in the presence of as little as 100 units of penicillin, it shifts to a resistant mode of reproduction, not inhibited by as much as 100,000 units of penicillin and capable of sustaining multiplication at one-half the normal rate.

However, the penicillin resistant feline pneumonitis virus is not completely insensitive to penicillin (figure 3). In this experiment, when increasing amounts of penicillin were given to one series of embryos immediately after virus inoculation, the resistant virus exhibited a constant growth rate over the whole range of antibiotic concentration. A second series of embryos received exactly corresponding quantities of virus and penicillin, but instead of being injected

separately, virus and drug were incubated together for two hours at 0 C before injection. The diverging curves relating average day of death to penicillin concentration indicate that contact with penicillin concentrations of 100,000 units per ml and greater progressively destroyed infectivity until complete inactivation occurred at 400,000 units. Although absurdly large amounts of penicillin are needed to inactivate the virus *in vitro*—400,000 units is 240 mg—the effect depends upon the intact penicillin structure because: first, penicillin inactivated by penicillinase has no such effect on the virus; and second, virus inactivated *in vitro* by penicillin may be reactivated by incubation with penicillinase. These observations suggest a direct interaction between virus and penicillin which should be amenable to further study.

The familiar phenomena of cross-resistance and collateral sensitivity may also be demonstrated with feline pneumonitis virus. After 44 passages on penicillin, the virus became more susceptible to chloramphenicol, and only half as much was required to give the same degree of protection against the penicillin resistant virus (unpublished observations). In a second series, when the parent virus was transferred 5 times in chick embryos given chloramphenicol, the resulting strain of virus had a heightened resistance to chlortetracycline although its susceptibility to chloramphenicol itself remained unchanged (unpublished observations). Despite the lack of any increased resistance to chloramphenicol, the frequent occurrence of cross-resistance between choramphenicol and chlortetracycline in bacteria (Herrell *et al.*, 1950; Pansy *et al.*, 1950) makes it likely that this is also an example of cross-resistance.

In the multiplication of any obligate intracellular parasite, the host cell makes a definite biochemical contribution toward the growth of the parasite so that their combined activities lead to reproduction of the parasite. The relative magnitude of this biochemical contribution of host and parasite varies greatly among different host-parasite systems. At one extreme, it is generally agreed that the bacterial viruses have no enzymes of their own and reproduce themselves by appropriating the enzymes of their hosts (Price, 1952). At the other, the malarial parasites possess such a wide variety of active enzymes that it is difficult to see why they are limited to an obligate intracellular existence (Moulder, 1948). Somewhere in between the two extremes are the rickettsiae, with limited, but very definite, enzymatic capabilities (Bovarnick and Snyder, 1949; Bovarnick and Miller, 1950).

Just where feline pneumonitis virus fits into this series remains to be determined. Its dependency on host function is shown by its inability to multiply in cells without an active aerobic metabolism. On the other hand, the chemical composition of the virus, its possession of both kinds of nucleic acid as well as lipid and protein-bound phosphorus, suggests a structural complexity compatible with independent enzymatic activity. This conclusion is supported by the susceptibility of the virus to chemotherapeutic agents known to inhibit bacterial enzyme systems. If feline pneumonitis virus does indeed have enzymes of its own, continued study of the chemical changes accompanying virus growth, of the effect of antibiotics and other metabolic inhibitors on multiplication, and of the biochemical properties of the isolated virus itself should lead inevitably to their discovery and thus to a better understanding of the basic nature of the psittacosis-lymphogranuloma group as a whole.

Finally, I am happy to acknowledge the major contributions of my students and associates to this work, especially those of Drs. Emilio Weiss, Stanley Zahler, Frank Gogolak and Arthur Brown.

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